

3. The following experiments were performed under my supervision.

a. Inhibition of Jurkat T cell leukemia and EL-4 T-cell lymphoma cellular proliferation by combinations of chemotherapeutic drugs and MCC

Human Jurkat T cell leukemia cells and murine EL-4 T cell lymphoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultivated as suspension cultures in RPMI 1640 cell culture medium supplemented with 10% heat-inactivated fetal calf serum and containing 50 µg/ml gentamycin sulfate (tissue culture medium: all from Sigma-Aldrich Canada, Oakville, Ontario). Cells were harvested by centrifugation (150×g for 15 minutes at 4°C), and resuspended in tissue culture medium to give a concentration of 1×10^5 cells/ml. Aliquots of this suspension (100 µl each) were placed in wells of 96-well tissue culture plates. The chemotherapeutic drugs cytosine arabinoside, daunorubicin, and mitoxantrone were obtained from Sigma-Aldrich Canada (Oakville, Ontario). The chemotherapeutic drugs were dissolved in tissue culture medium, sterile filtered, diluted, and added to a sterile suspension of MCC in tissue culture medium (0.1 µg/ml) to give the final drug concentrations of 0.001-1.0 µg/ml. 100 µl drug/MCC combination were then added to the cells, and incubation was carried out for 48 hours at 37°C in an atmosphere of 5.0% CO₂/95% air. At the end of the incubation, inhibition of cellular proliferation was determined by dimethylthiazoldiphenyltetrazolium (MTT) reduction (Mosman *et al.*, *Journ. Immunol. Methods* 65:55, 1983). The potency of the chemotherapeutic agent plus MCC was compared to the chemotherapeutic agent alone using software Pharm/PCS version 4.2 (Computer Associates, Philadelphia, PA, USA).

The concentration of MCC used in these studies was determined to give between 5-10% inhibition of proliferation in the absence of chemotherapeutic agents.

The concentrations of chemotherapeutic drugs used (0.001-1.0 µg/ml) were determined to give between 10 and 90% inhibition of cellular proliferation. The potency of the chemotherapeutic agents in combination MCC relative to the chemotherapeutic agents alone is shown in Table 1. The results show that the addition of sub-optimal amounts of MCC to cytosine arabinoside, daunorubicin or mitoxantrone significantly increased the potency of these drugs when compared to cytosine arabinoside, daunorubicin or mitoxantrone alone.

Table 1

MCC enhances the potency of chemotherapeutic agents

Chemotherapeutic agent	Relative potency (95% Confidence Limits)			
	Jurkat T cell leukemia		EL-4 T cell lymphoma	
	No MCC	+MCC	No MCC	+MCC
Cytosine arabinoside	1.0	8.4 (1.9-55)	1.0	5.3 (2.0-16.9)
Daunorubicin	1.0	4.0 (0.6-81.0)	1.0	6.0 (2.0-19.0)
Mitoxantrone	1.0	4.4 (1.5-32)	1.0	7.2 (2.0-45.0)

These results demonstrate that the combination of MCC with chemotherapeutic agents significantly increased their potency against leukemia and lymphoma cells.

b. MCC potentiation of the antiproliferative activity of mitoxantrone on prostate LNCaP cancer cells

The prostate LNCaP cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). LNCaP cells were cultured in RPMI 1640 cell culture medium containing 10% heat-inactivated fetal bovine serum and 50 µg/ml gentamycin (all from Sigma-Aldrich Canada, Oakville, Ontario) at 37°C in an atmosphere of 5.0% CO₂/95% air. LNCaP cells were incubated in individual wells of 24-well plates at 2.0 x 10⁵ cells/ml for 72 hours with saline, 0.1

$\mu\text{g/ml}$ of MCC formulated in hyaluronic acid (1:1 w/w, the hyaluronic used has a molecular weight average of 750 kDa and was provided by Bioniche Life Sciences Inc (Belleville, Ontario, Canada)) and/or 0.5, 5.0 and 50.0 $\mu\text{g/ml}$ of mitoxantrone. Mitoxantrone was obtained from Sigma-Aldrich Canada. Cell proliferation was measured using dimethylthiazoldiphenyltetrazolium (MTT) reduction (Mosman *et al. Journ. Immunol. Methods* 65:55, 1983). Table 2 shows the percentage of inhibition of cell proliferation.

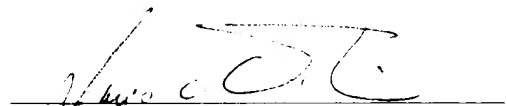
Table 2

MCC potentiates the antiproliferative of mitoxantrone

	MITOXANTRONE			
	0.0 $\mu\text{g/ml}$	0.5 $\mu\text{g/ml}$	5.0 $\mu\text{g/ml}$	50.0 $\mu\text{g/ml}$
Saline +	0.0%	0.0%	0.0%	7.1%
MCC-HA (0.1 $\mu\text{g/ml}$) +	19.5%	34.9%	41.1%	44.4%

As shown in Table 2, MCC formulated with hyaluronic acid potentiated the antiproliferative of mitoxantrone towards prostate LNCaP cancer cells.

5. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any patent issuing on this application.


Mario C. Filion, Ph.D.


Date

April 15, 2003